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IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT: Ichiro Azuma, et al.

SERIAL NO: 09/743,750 GROUP: 1645

FILED: January 16, 2001 EXAMINER: Vanessa L. Ford

FOR: FORMULATIONS USEFUL FOR IMMUNOTHERAPY FOR CANCERS CONTAINING BACTERIAL COMPONENT AS AN ACTIVE INGREDIENT

DECLARATION UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Takehiko Nomura, a citizen of Japan and residing at Osaka, Japan, say and declare as follows:

1. I received the degree of Ph. Dr. from Kyoto University in Japan in 1998.

2. I have been working at Sumitomo Pharmaceuticals Research Center since 1998, studying pharmaceutical science and drug formulation.

3. I am a member of Parenteral Formulation Research Group in Formulation Research Laboratories.

4. I am an author or co-author of 3※ papers related to pharmacetucial science and cancer gene therapy.

※Pharm Res. 1998 Jan;15(1):128-32.
J Control Release. 1998 Mar 31;52(3):239-52
Cancer Res. 1997 Jul 1;57(13):2681-6.

5. Although I am not one of the inventors in U.S. Serial Number 09/743,750, I am very familiar with the subject matter thereof and have been researching the subject matter thereof since 1998.

6. I have conducted the following experiments related to the subject matter of the 09/743,750 application.

7. Comparative Experiment

The comparative experiment is to demonstrate the difference in particle size of the particle of BCG-CWS when a dispersion-aiding solvent is used or not.

MATERIALS AND METHODS

The emulsions of the oil droplets comprising BCG-CWS were prepared according to the procedures described in the specification of the present application, U.S. Patent Application No. 09/743,750.

Experiment 1

(a)BCG-CWS (163 mg) was added to 10 ml of a solvent of 10% ethanol and 90% heptane, and the mixture was dispersed via sonication at room temperature for 30 minutes. Then, squalane (2.1 g) was added to the dispersion, and the mixture was mixed, and heated at 60-70 °C under a flow of nitrogen with stirring to evaporate the solvent, thus obtaining an oil.

(b)BCG-CWS (210mg), squalane (2.64g) and 10ml of toluene were mixed, and the mixture was dispersed via sonication at room temperature for 30 minutes. Then, the mixture was heated at 70 °C under a flow of nitrogen with stirring to evaporate the solvent, thus obtaining an oil.

Experiment 2

BCG-CWS (163 mg) and squalane (2.1 g) were mixed together, and the mixture was dispersed via sonication at room temperature for 30 minutes.

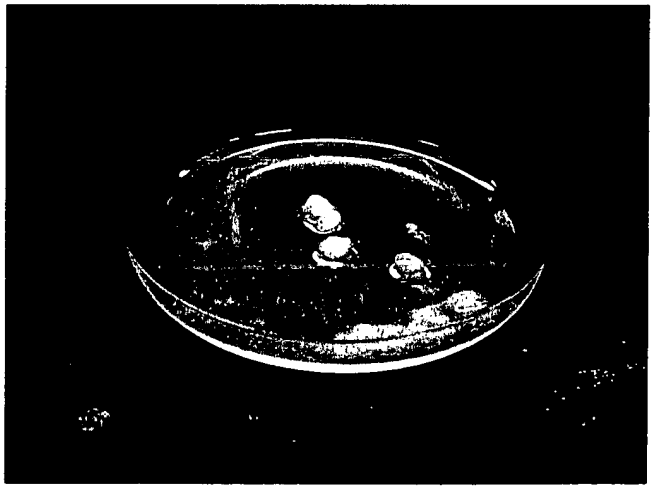
RESULTS

First, the appearance of BCG-CWS as used in Experiments 1 and 2 is shown in the following:

The appearance of the suspensions as obtained in Experiments 1 and 2 are shown in the following:

Experiment 1(a)

Experiment 2



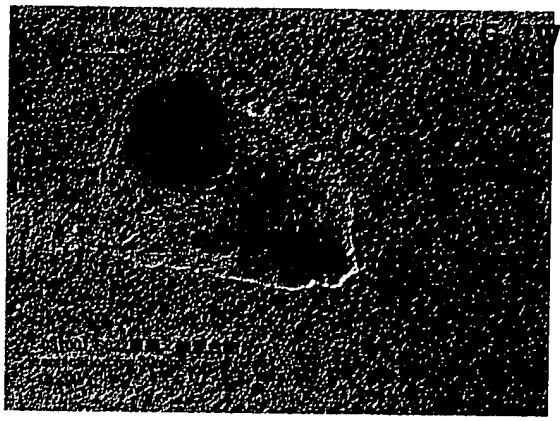
Experiment 1 provided homogenous particles of BCG-CWS, whereas Experiment 2 gave a mixture of the large mass of BCG-CWS particles.

Photomicrographs of the oils comprised in the suspensions as

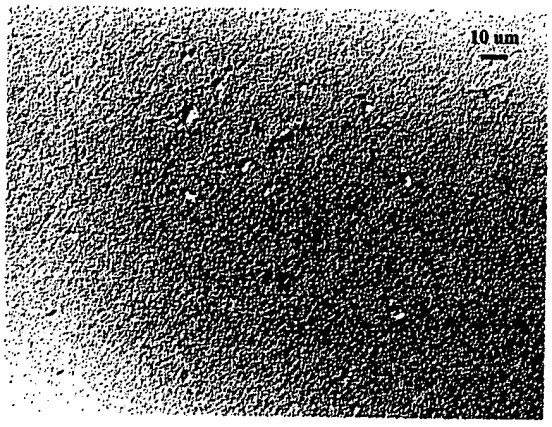
obtained by Experiments 1 and 2 are shown in the following:

Experiment 1 (a)

Experiment 2



Experiment 1 (b)



Experiment 1(a) and 1(b) provided finely homogenous microparticles, whereas Experiment 2 gave a mixture of a particle having a large particle size.

8. The undersigned declares further that all statement made herein of his own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that Such willful false statement may jeopardize the validity of above identified application or

any patent issuing thereon.

29. June 2004

Date

Takehiko Nomura

Dr. Takehiko Nomura

IN THE U.S. PATENT AND TRADEMARK OFFICE

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DECLARATION UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Keigo Kawabe, a citizen of Japan and residing at Osaka, Japan, say and declare as follows:

1. I received the degree of Ph. Dr. from Osaka Medical College in Japan in July 23, 1997.

2. I have been working at Sumitomo Pharmaceuticals Research Center since 1991, studying virology, biotechnology products and electron microscopy.

3. I am a member of the medical society of Osaka Medical College.

4. I am an author or co-author of the following 4 papers related to virology.

1) Kawabe K., J. Osaka Med. Coll. 56(1): 37-44, 1997, "In vitro antiviral effects of natural alpha interferon on human immunodeficiency virus type 1"
2) Sano K, Takasaki T, Fukui A, Jiang Y, Urabe T, Nakano T, Saito

Y, Nakamura T, Kise M, Kawabe K, et al., Journal of the Japanese Association for Infectious Diseases (J J A Inf D) 67(5): 459-65, 1993, "A stick type filter unit for prevention of biohazard in a research laboratory"

3) Kawabe K, Morimatsu S, Nakano T, Goto T, Nakai M, Proceedings of the sixth asia-pacific conference on electron microscopy: 475-476, 1996, "In vitro antiviral effects on human immunodeficiency virus type1 by alpha interferon"

4) Goto T, Nakano T, Morita C, Kakimoto K, Hong W, Kawabe K, et al., Proceedings of the sixth asia-pacific conference on electron microscopy: 473-474, 1996, "Detection of Nucleic acids in human immunodeficiency virus particles by electron microscopic in situ hybridization".

5. Although I am not one of the inventors in U.S. Serial Number 09/743,750, I am very familiar with the subject matter thereof and have been researching the subject matter thereof since December 18, 1998.

6. I have conducted the following experiments related to the subject matter of the 09/743,750 application.

7. The form of BCG-CWS in organic solvents

The following three (3) experiments demonstrate that the morphology of BCG-CWS treated with an organic solvent is quite different from that of BCG-CWS treated with a physiological saline as commonly used.

8. Ultrathin Sectioning

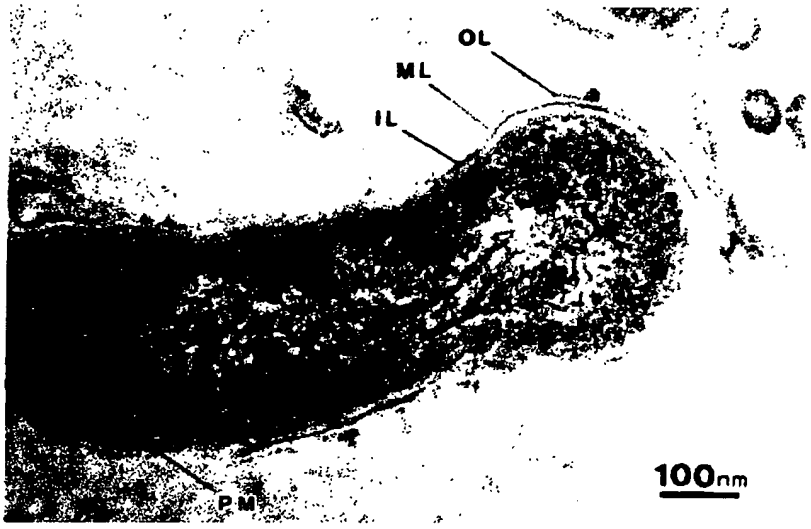
Viable BCG and BCG-CWS were suspended in a physiological saline or toluene, and fixed in 5% acrolein. The solvents were then replaced with a cacodylate buffer, and an additional fixing was conducted in 1% osmic acid. After dehydration with acetone, the materials were embedded into the Spurr resin, and cut off to give the

sections of 30-80 mm in thickness. The sections were double-stained with uranyl acetate and lead, and observed with a transmission electron microscope (TEM).

RESULTS

First, the morphology of the viable BCG is shown in Figure 1 to illustrate the structure of the cell wall skeleton (CWS).

Figure 1 (image of viable BCG)

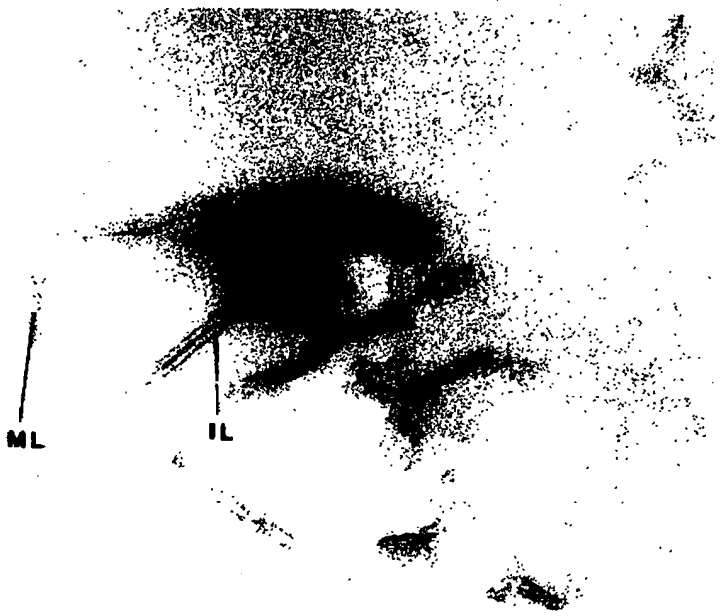


In Figure 1, "OL", "ML", "IL", and "PM" represent the outer membrane, the mycolic acid layer, the peptideglycan layer, and the plasma membrane, respectively. In the viable BCG, the mycolic acid and the peptideglycan are arranged in layers, said mycolic acid layer being lower in electron density (stained thinly), whereas said peptideglycan layer being higher in electron density (stained densely).

Next, the morphologies of BCG-CWS derived from viable BCG that are suspended in a physiological saline and a toluene are illustrated.

The morphology of the BCG-CWS suspended in the physiological saline is shown in Figure 2, wherein "ML" is the mycolic acid layer and "IL" is the peptideglycan layer. The mycolic acid layer (stained thinly) existed inside, and the peptideglycan layer (stained densely) was exposed outside, showing that the BCG-CWS was in the flat and folded form.

Figure 2 (BCG-CWS in a physiological saline)



On the other hand, the BCG-CWS suspended in toluene assumed the morphology as shown in Figure 3 in which the mycolic acid was exposed outside, showing that the BCG-CWS was in the elongated and rolled membrane-like form.

Figure 3 (BCG-CWS in toluene)

9. Histochemical Staining

The BCG-CWS which had been suspended in a physiological saline or toluene was fixed in 2% glutaraldehyde, and the solvents were then replaced with PBS(-). After dehydration with ethanol or acetone, the materials were embedded into Lowicryl K4M resin, and cut off to give the sections of 50-80 mm in thickness. The sections were subjected to the histochemical staining with N- acetyl glucosamine-specific lectin: WGA. Specifically, a lectin that bond selectively to N-acetyl glucosamine, WGA, was conjugated with a gold colloid and the conjugate was used to stain the sections, after which the localization of the gold colloid was observed using a transmission electron microscope.

RESULTS

Figure 4 reveals that, when the BCG-CWS was suspended in a physiological saline, the peptideglycan layer (IL: stained densely) which was localized the gold colloid existed outside, and the mycolic acid layer (ML: stained thinly) existed inside.

On the other hand, when the BCG-CWS was suspended in toluene, the mycolic acid was found to be exposed outside and rolled as shown in Figure 5. The morphologies were similar to those shown in Section 8 above.

Figure 4 (in physiological saline)

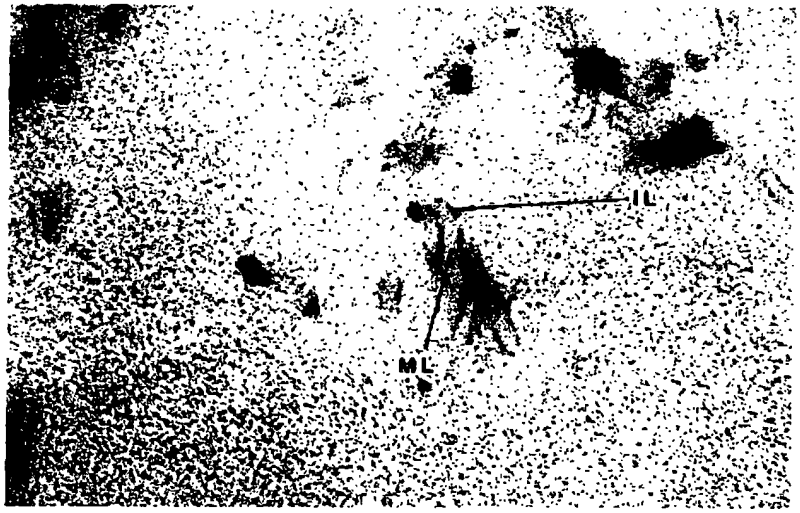
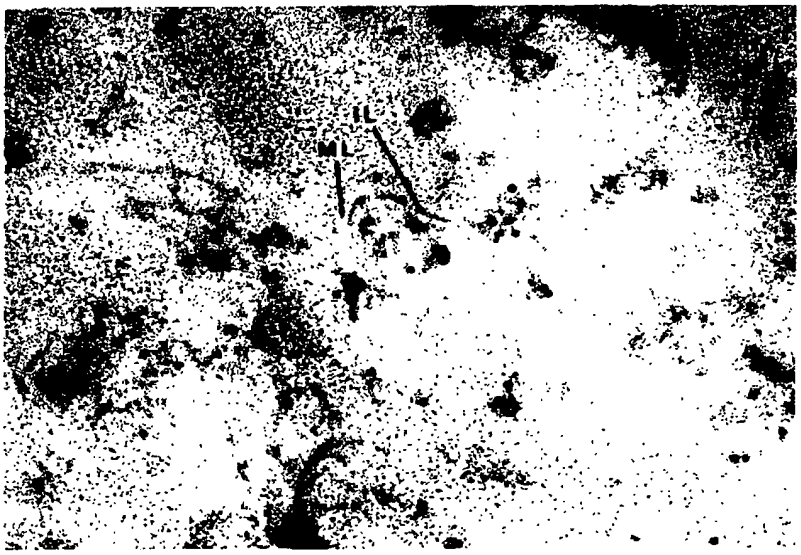


Figure 5 (in toluene)



10. Negative staining

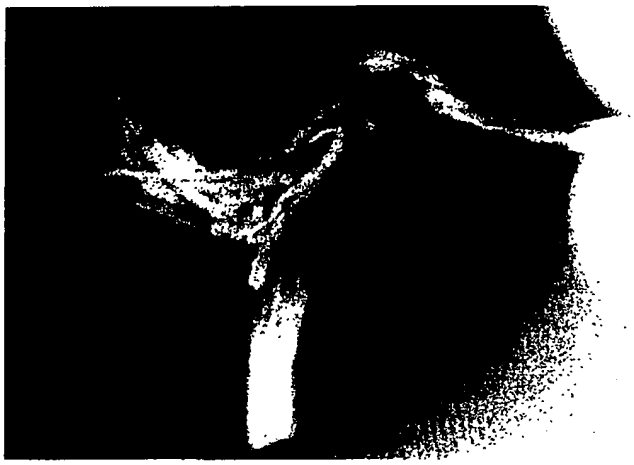
The BCG-CWS was suspended in a physiological saline or toluene, and the suspension was dropped onto an observation grid and fixed. The materials was subjected to electron staining with phosphotungstic acid, and observed using a transmission electron microscope.

RESULTS

In this experiment, the entire morphology of BCG-CWS was observed at low magnification. The observation with negative staining revealed that the entire BCG-CWS suspended in a physiological saline resulted in fragments that assumed the flat or folded forms, whereas the BCG-CWS suspended in toluene gave fragments that assumed the vorticosely rolled forms. Thus, those morphologies were different each other.

Figure 6: Physiological Saline

Figure 7: Toluene



11. The undersigned declares further that all statement made herein of his own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that Such willful false statement may jeopardize the validity of above identified application or any patent issuing thereon.

July 7, 2004
Date

Keigo Kawabe
Dr. Keigo Kawabe